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Characterization of the cleavage site and the recognition sequence of the I-*Cre*I DNA endonuclease encoded by the chloroplast ribosomal intron of *Chlamydomonas reinhardtii*

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Summary. The chloroplast ribosomal intron of *Chlamydomonas reinhardtii* encodes a sequence-specific DNA endonuclease (I-*Cre*I), which is most probably involved in the mobility of this intron. Here we show that I-*Cre*I generates a 4 bp staggered cleavage just downstream of the intron insertion site. The I-*Cre*I recognition sequence is 19–24 bp in size and is located asymmetrically around the intron insertion site. Screening of natural variants of the I-*Cre*I recognition sequence indicates that the I-*Cre*I endonuclease tolerates single and even multiple base changes within its recognition sequence.

Key words: *Chlamydomonas* – Cleavage site – DNA endonuclease – Group I intron – Recognition sequence

Introduction

Several group I introns are known to be mobile genetic elements. Mobile introns have been found in nuclear, mitochondrial, chloroplast and bacteriophage genomes (for review see Dujon 1989; Perlman and Butow 1989). Mobile introns are transmitted with very high frequency to all the progeny of genetic crosses between intron-containing and intron-deficient parents. This intron transmission is also called "intron homing" (Dujon et al. 1989). All mobile introns encode sequence-specific DNA endonucleases. These enzymes generate a staggered double-strand cleavage in the recipient (intronless) allele in the vicinity of the intron insertion site. This double-strand break is thought to initiate a recombination event, which leads to the replicative integration of the intron into the intronless allele (Colleaux et al. 1988). Mobility of the intron in the chloroplast 23S ribosomal RNA gene of *Chlamydomonas reinhardtii* has been demonstrated using chloroplast transformation. When a partial 23S cDNA spanning the intron insertion site was stably integrated at an ectopic position in the chloroplast genome, all the transformants with a correctly integrated 23S cDNA acquired a new copy of the ribosomal intron at the ectopic position (Dürrenberger and Rochaix 1991).

The chloroplast ribosomal intron of *C. reinhardtii* contains an internal open reading frame (ORF) that encodes a predicted polypeptide of 163 amino acid residues (Rochaix et al. 1985). Using an in vivo assay system in *Escherichia coli* with two plasmids, one expressing the ribosomal intron ORF and the other carrying the 23S cDNA as a substrate, we have previously demonstrated that this ORF encodes the I-*Cre*I DNA endonuclease which produces a double-strand cleavage close to the exon junction of the 23S cDNA (Dürrenberger and Rochaix 1991). Here we show that the I-*Cre*I endonuclease generates a 4 bp staggered cleavage resulting in 4-base protruding 3′ ends of the cleavage products. The cleavage site is located downstream of the intron insertion site. The recognition sequence lies within a stretch of 19–24 bp, spanning the intron insertion site and the cleavage site. Furthermore, cleavage reactions using as substrates cloned large subunit (LSU) ribosomal DNAs from different organisms indicate that the I-*Cre*I endonuclease tolerates single and even multiple changes within its recognition sequence.

Materials and methods

Plasmids. pORF, containing the open reading frame (ORF) of the *C. reinhardtii* ribosomal intron (I-*Cre*I gene) in the pUHE25-1 expression vector, was used for expression of I-*Cre*I is *E. coli*. pc23S is a pACYC184 derivative containing the I-*Cre*I recognition site within a 620 bp *C. reinhardtii* 23S ribosomal cDNA. Construction of pORF and pc23S have been described previously (Dürrenberger and Rochaix 1991). pKS:c23Su and pKS:c23Sl were constructed by cloning the 620 bp 23S cDNA (purified from a polymerase chain reaction) in both orientations in the *Bam*HI site of pKS$^-$ (Stratagene). pKS:c23Su and pKS:c23Sl direct the production of single-stranded DNA phages containing the upper strand and the lower strand (see Fig. 1C) of the 23S

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cDNA insert, respectively. The following plasmids were used as substrates for I-*Cre*I cleavage: pc23S; p16-3 (oligonucleotide linker containing 31 bp of the 23S cDNA of *C. reinhardtii*, inserted in the *Sal*I site of pACYC*lacI*^{q}; Fassbender and Kück, unpublished); pKW223 (*Bacillus stearothermophilus;* Kop et al. 1984); pCCE100 (*Chlorella ellipsoidea*, chloroplast; Yamada and Shimaji 1986); pKK3535 (*E. coli;* Brosius et al. 1981); pMYC501 (*Mycoplasma* PG50; Rasmussen and Christiansen 1987); restriction fragment 4E3 in pBR325 (*Paramecium* sp. 4 strain 51, mitochondria; Seilhamer et al. 1984); pUC35 (*Rhodobacter sphaeroides;* Dryden and Kaplan 1990); pSCM311 (*Saccharomyces cerevisiae*, mitochondria; Dujon, unpublished); and pSP6/Zmc23SHK (*Zea mays*, chloroplast; Delp and Kössel, unpublished).

Preparation of extracts from E. coli *cells expressing* I-*Cre*I. An overnight culture of XL-1 blue (Stratagene) transformed with pORF was diluted 50-fold in fresh LB medium containing 50 µg/ml ampicillin. After 2 h of incubation at 37° C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM. Cells were harvested after an additional incubation of 2 h at 37° C. Cells were resuspended in 1/20th volume of extraction buffer [20 mM TRIS–HCl, pH 7.4; 15 mM NaCl; 10% glycerol; 1 mM EDTA; 0.1 mM phenylmethylsulphonyl fluoride (PMSF)], lysozyme was added to 0.1 mg/ml and the cells were left on ice for 30 min. Cells were disrupted by a 10 min sonication (Branson Sonifier B12, microtip, 25 W). The lysate was spun for 5 min in a microcentrifuge and the supernatant was heated for 10 min to 60° C to inactivate most of the contaminating DNA nuclease activity present in the extract, whereas I-*Cre*I activity was found to be resistant to this heat treatment. Denatured proteins were removed with a 10 min centrifugation in a microcentrifuge; the supernatant was recovered and stored at 4° C.

I-*Cre*I *cleavage reactions*. CsCl purified DNA substrates were digested with appropriate restriction enzymes (pc23S with *Nco*I; pKK3535 with *Bam*HI; all the other plasmids with *Eco*RI), extracted with phenol, end-labelled using Klenow DNA polymerase and [^{32}P]dATP, precipitated and resuspended in TE (10 mM TRIS–HCl, pH 8.0; 1 mM EDTA). Samples of the labelled substrates were electrophoresed on agarose gels and the amount of DNA was quantified by ethidium bromide staining to ensure that equal amounts of substrate DNAs were added to the cleavage reactions. Approximately 10 ng of end-labelled, linear substrate DNA and a minimal amount of freshly diluted extract (usually corresponding to 0.5 µl undiluted extract) were added to a total volume of 20 µl (20 mM TRIS–HCl, pH 7.4; 10 mM $MgCl_2$). Cleavage reactions were carried out for 60 min at 37° C and stopped by heating to 68° C for 15 min. Extracts were always titrated, using pc23S as a substrate, in order to determine the minimal amount of extract needed for maximal cleavage. I-*Cre*I activity of these extracts varied from batch to batch and it was sometimes not possible to obtain complete cleavage of the pc23S substrate.

Mapping of the cleavage site and the recognition sequence of I-*Cre*I. Single-stranded DNAs of pKS:c23Sl and pKS:c23Su were prepared and converted into their double-stranded form as follows. Approximately 1 µg of single-stranded pKS:c23Sl and pKS:c23Su was annealed to 0.5 pmol of ^{32}P kinase-labelled oligonucleotide 5′23S.2 and 3′23S.2, respectively. 5′23S.2 and 3′23S.2 hybridize ~100 nucleotides upstream and downstream (see Fig. 1C), respectively, of the intron insertion site (Dürrenberger and Rochaix 1991). Primer extensions were carried out in the presence or absence of dideoxy nucleotides, following the Sequenase protocol (United States Biochemical Corporation) with the modification that no labelled dATP was present. The products of the extension reactions were cleaved with 8 µl of I-*Cre*I extract in a total volume of 50 µl, phenol extracted, precipitated and resuspended in loading buffer. To map the cleavage sites in the upper and lower strand (see Fig. 1C), the cleavage products of the extension reactions primed with 5′23S.2 and 3′23S.2, respectively, were run on a sequencing gel alongside the corresponding dideoxy terminated reactions. The products of the sequencing reactions were cleaved with the same I-*Cre*I extract. The amount of extract required to achieve complete digestion of the products of the sequencing reactions primed with 5′23S.2 was increased 15-fold compared to the products of the 3′23S.2 primed reactions. To map the right and the left boundary (see Fig. 2C) of the I-*Cre*I recognition sequence, the cleaved products of the sequencing reactions primed with 5′23S.2 and 3′23S.2, respectively, were separated on a sequencing gel in parallel with the uncleaved products of the sequencing reactions.

Results

Cleavage site and recognition sequence of I-*Cre*I

The cleavage site and the maximal boundaries of the recognition sequence were determined essentially as described by Wenzlau et al. (1989). To map the cleavage site of I-*Cre*I on the 23S cDNA, double-stranded substrate DNAs having either the upper or the lower strand (see Fig. 1C) radioactively labelled were prepared as follows. Single-stranded DNAs from plasmids that contain the 23S cDNA in both orientations were used as templates for complementary strand DNA synthesis, which was primed by ^{32}P-labelled oligonucleotides hybridizing approximately 100 bases upstream or downstream (see Fig. 1C) of the intron insertion site. These substrate DNAs were incubated with an extract prepared from *E. coli* cells expressing I-*Cre*I activity from the plasmid pORF. Although most contaminating DNA nuclease activities could be inactivated by heating the extract to 60° C, the I-*Cre*I activity is resistant to this heat treatment (data not shown). No I-*Cre*I endonuclease activity could be detected in heat-treated extracts prepared from *E. coli* cells containing the expression vector with the I-*Cre*I coding sequence inserted in the antisense orientation (data not

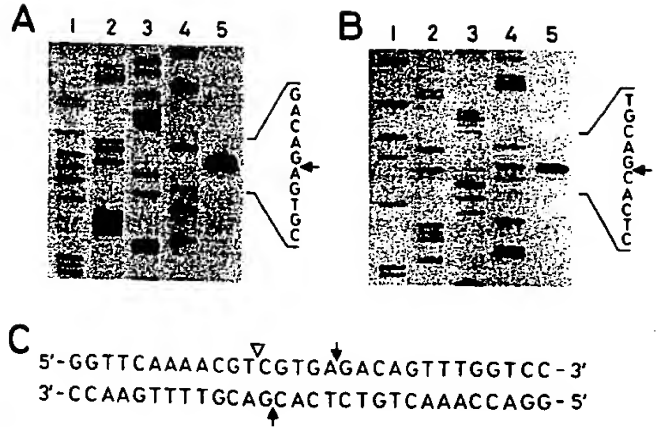

Fig. 1A–C. Cleavage site of I-*Cre*I: mapping of the cleavage site of I-*Cre*I in **A** upper and **B** lower strand of *Chlamydomonas reinhardtii* 23S cDNA. The upper or the lower strand of substrate DNAs bearing the 23S cDNA were end-labelled and incubated with extract prepared from bacteria expressing I-*Cre*I. Products of the cleavage reactions were separated on a denaturing polyacrylamide gel together with the corresponding sequencing reactions (lanes 1–4; dideoxy G, A, T and C, respectively). Sequences around the cleavage site are indicated; *arrows* point to the position where cleavage occurs. **C** The sequence flanking the intron insertion site *(open triangle)* is shown and cleavage sites on both strands are indicated by *arrows*

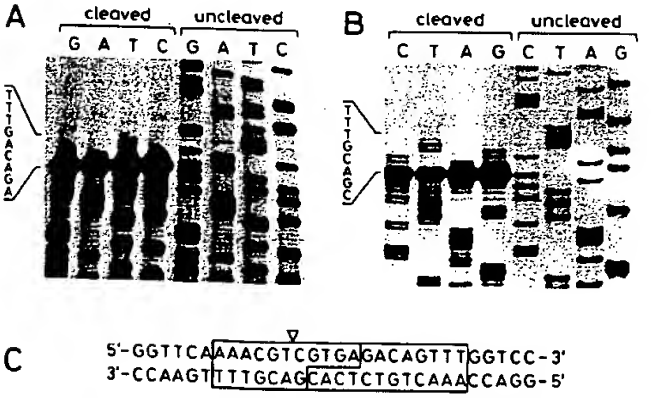

Fig. 2. Boundaries of the I-*Cre*I recognition sequence: mapping of **A** right boundary and **B** left boundary of the recognition sequence. The products of the sequencing reactions shown in **A** and **B** ("uncleaved") were incubated with the extract from *Escherichia coli* cells expressing I-*Cre*I. Cleaved and uncleaved sequencing reaction products were resolved on a denaturing polyacrylamide gel. The strong bands in **A** "cleaved" and **B** "cleaved" correspond to the cleavage products shown in lane 5 of Fig. 1A and B, respectively. The faint bands above the strong bands correspond to the labelled strands of the partially double-stranded DNA substrates, which are resistant to cleavage, and thereby delimit the recognition sequence. **C** The 23S sequence surrounding the intron insertion site *(open triangle)* is shown. The *staggered line* indicates the cleavage site; the proposed recognition sequence is *boxed*

shown). The cleavage reaction products were run on a sequencing gel with the corresponding sequencing ladders (Fig. 1A and B). As summarized in Fig. 1C, I-*Cre*I cleaves the upper strand 5 bases downstream and the lower strand 1 base downstream of the intron insertion site. This results in a 4 bp staggered cut generating 3′ overhanging ends.

To delineate the recognition sequence of I-*Cre*I, the substrates for the cleavage reactions were synthesized in the presence of dideoxy nucleotides. The partially double-stranded DNA molecules, having a recognition sequence that has been converted completely to the double-stranded form, are cleaved by the enzyme. The substrate molecules generated by chain termination at one of the nucleotide positions within the recognition sequence are resistant to cleavage and thereby delimit the recognition sequence. To map the left boundary of the recognition sequence, the lower strand was synthesized (Fig. 2B). The bands corresponding to the resistant substrate DNA molecules indicate that 6 bp located 5′ to the intron insertion site are sufficient for cleavage (Fig. 2C). The additional bands present in the sequencing ladders of the cleaved samples are probably due to slight exonucleolytic degradation caused by contaminating exonuclease activity present in the heat-treated extract. To map the right boundary of the recognition sequence, the upper strand was synthesized (Fig. 2A). The result is not as clear as for the left boundary, since cleavage of these substrate DNAs was not as efficient as for the left boundary, although fifteen times more extract was used. Therefore the right limit of the recognition sequence, drawn at 13 bp 3′ from the intron insertion site (Fig. 2C), may extend a few base pairs beyond this point.

Effect of naturally occurring variant recognition sequences on cleavage efficiency of I-*Cre*I

Cleavage reactions were performed using as substrates a set of subclones of ribosomal LSU genes isolated from different organisms. The variant I-*Cre*I recognition sequences contained within these ribosomal DNAs are aligned with the *C. reinhardtii* sequence in Fig 3A. Cleavage of these heterologous substrates was compared to cleavage of the *C. reinhardtii* 23S cDNA and of a synthetic 23S cDNA sequence of 31 bp spanning the intron insertion site (C.r.oligo). The size of the I-*Cre*I cleavage products of the different ribosomal DNAs was consistent with cleavage at the variant I-*Cre*I recognition sites.

Surprisingly, the 31 bp 23S cDNA sequence is cleaved with higher efficiency than the original 620 bp 23S cDNA (Fig 3B, lanes 2 and 3). Even more surprising is the finding that the *Mycoplasma* clone appears to be a better substrate than the 620 bp *C. reinhardtii* cDNA (lane 4). The *Mycoplasma* ribosomal DNA is cleaved with the same efficiency as the synthetic 31 bp 23S cDNA sequence from *C. reinhardtii*, although the *Mycoplasma* clone has a one base substitution (A→G) at the left boundary of the recognition sequence (Fig. 3A). All the ribosomal clones that have, in addition to the change present in *Mycoplasma*, the same second change at the right boundary of the recognition sequence (T→C) are cleaved with lower efficiency compared to the 31 bp 23S cDNA sequence (Fig. 3B; lanes 5–8, "G+C" variants). The *Paramecium* clone, which has a single base change

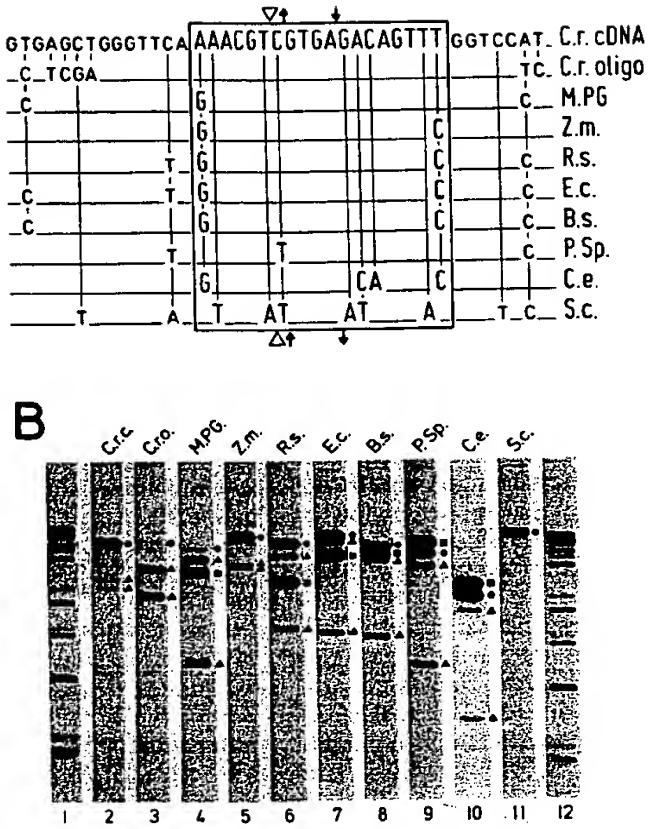

Fig. 3A, B. Cleavage of natural variants of the I-*Cre*I recognition sequence. **A** Sequence alignment of the different I-*Cre*I recognition sites *(larger letters)* and flanking sequences *(smaller letters)*. The entire sequence is given only for the *C. reinhardtii* 620 bp 23S cDNA (C.r.cDNA). For the 31 bp 23S cDNA sequence (C.r.oligo) and the different variants only nucleotides deviating from the *C. reinhardtii* sequence are indicated. The intron insertion site is indicated by an *open triangle* and positions of the cleavage in the upper (↓) and lower strand (↑) are indicated by *arrows*. Ribosomal LSU genes from the following organisms were used: *Bacillus stearothermophilus* (B.s.), *Chlorella ellipsoidea* (C.e.; chloroplast), *E. coli* (E.c.), *Mycoplasma* PG50 (M.PG), *Paramecium* sp. 4 (P.Sp; mitochondria), *Rhodobacter sphaeroides* (R.s.), *Saccharomyces cerevisiae* (S.c.; mitochondria), *Zea mays* (Z.m.; chloroplast). **B** Cleavage reactions. The substrate plasmids were either linearized (lanes 2, 3, 5 and 11) or the ribosomal inserts were cut out (lanes 4 and 6–10) with appropriate restriction enzymes and end-labelled. The cleavage reactions consisted of ~10 ng of substrate DNA and an equal amount of extract from bacteria expressing I-*Cre*I. The products of the cleavage reactions (lanes 2–11) were resolved on a 1% agarose gel. The origin of each substrate DNA is indicated *above* each lane (C.r.c., *C. reinhardtii* 620 bp 23S cDNA; C.r.o., *C. reinhardtii* 31 bp cDNA sequence). Bands corresponding to linear ribosomal substrate DNAs *(circles)*, cleavage products *(triangles)* and plasmid vectors *(squares)* are indicated. The expected sizes of the I-*Cre*I cleavage products are 2.6 and 2.3 kb for C.r.c.; 2.9 and 2.1 kb for C.r.o.; 3.7 and 0.9 kb for M.PG.; 3.5 and 3.4 kb for Z.m.; 4.1 and 1.5 kb for R.s.; 6.1 and 1.4 kb for E.c.; 3.7 and 1.0 kb for B.s.; 3.2 and 1.2 kb for P.Sp.; 2.0 and 0.3 kb for C.e. Lanes 1 and 12, DNA size standards (lambda DNA digested with *Bst*EII: 8.5, 7.2, 6.4, 5.7, 4.8, 4.3, 3.7, 2.3, 1.9, 1.4, 1.3, 0.7, 0.2 and 0.1 kb)

at the position corresponding to the first base of the 3′ exon (C→T) is cleaved with an efficiency comparable to that observed for the "G+C" variants (lane 9). The *Chlorella* recognition sequence which has four bases changed is still cleaved to some extent (lane 10), whereas the yeast mitochondrial clone containing a variant recognition sequence with six substitutions is the only ribosomal clone that is not cleaved at all (lane 11).

Discussion

The cleavage site of the I-*Cre*I endonuclease encoded by the *C. reinhardtii* chloroplast ribosomal intron was determined using a heat-treated extract from bacteria expressing the I-*Cre*I enzyme. The cleavage site was found to map 5 bases and 1 base downstream of the intron insertion site on the rRNA-like and its complementary strand, respectively. I-*Cre*I cleavage generates 4-nucleotide overhanging 3′ ends and behaves in this respect like the other five characterized intron-encoded DNA endonucleases described from mitochondria of *S. cerevisiae* (I-*Sce*I, *I-Sce*II), nuclei of the slime mold *Physarum polycephalum* (I-*Ppo*I), the archebacterium *Thermococcus litoralis* (I-*Tli*I; Perler et al. in preparation) and from the *C. eugametos* chloroplast (I-*Ceu*I). The positioning of the I-*Cre*I cleavage site with respect to the intron insertion site is the same as those found for I-*Ceu*I (Marshall and Lemieux 1991) and I-*Sce*II (Wenzlau et al. 1989; Delahodde et al. 1989) and shifted downstream by only 2 and 3 bp when compared to I-*Ppo*I (Muscarella et al. 1990) and I-*Sce*I (Colleaux et al. 1988), respectively. The I-*Cre*I endonuclease generates a 4 bp staggered cut similar to the eukaryotic and the archebacterial "homing endonucleases", whereas the T4 phage enzymes I-*Tev*I and I-*Tev*II cleave in a 2 bp staggered fashion. Furthermore, the phage endonucleases cleave their target sequences at an increased distance (for I-*Tev*II, 13–15 bp and for I-*Tev*I, 23–25 bp) from the intron insertion sites (Bell-Pedersen et al. 1990; Chu et al. 1990) compared to the other examples.

The recognition sequence of I-*Cre*I was delimited using the method described by Wenzlau et al. (1989). This method gives an upper limit for the size of the recognition sequence, because it is based on cleavage by the enzyme at the end of double-stranded DNA. It is well known that most type II restriction enzymes do not efficiently cleave recognition sequences localized at the very end of a DNA fragment. Therefore it is possible that the left border of the I-*Cre*I recognition sequence lies a few bp further downstream (see Fig. 2C), when embedded in a larger DNA fragment. The result obtained with the *Mycoplasma* ribosomal clone supports this notion, since the A→G change at the left end of the *Mycoplasma* recognition sequence does not effect cleavage efficiency. The determination of the position of the right boundary of the recognition sequence is somewhat more ambiguous, because the substrate DNAs used for mapping the right boundary were more refractory to I-*Cre*I cleavage than those used to map the left boundary and consequently

more extract had to be used to obtain comparable cleavage. This apparent difference in cleavage efficiency for the partially double-stranded substrates used for the mapping of the two extremities of the recognition sequence could be due to differences in the ability of the single-stranded sequences at the left and at the right boundaries to form secondary structures, which might interfere with I-*Cre*I binding or cleavage activity. However, the T→C change at the proposed right end of the recognition sequence present in several ribosomal clones (see Fig. 3) reduces cleavage efficiency, thereby confirming that the recognition sequence extends at least to this point. Furthermore, the fact that the 31 bp cDNA is efficiently cleaved demonstrates that the complete recognition sequence is contained within these 31 bp. This 31 bp cDNA extends 5 bp beyond the proposed right boundary of the recognition sequence (see Fig. 3A) and therefore positions the upper limit for the right boundary only 5 bp downstream of the proposed right boundary. Taken together, this demonstrates that I-*Cre*I recognizes a sequence of 19–24 bp starting, at the most, 6 bp upstream of the intron insertion site and ending, at the most, 18 bp downstream of this position.

The finding that the 31 bp 23S cDNA sequence is a much better substrate for I-*Cre*I than the 620 bp cDNA suggests the presence of a DNA sequence element within the 620 bp cDNA, which reduces I-*Cre*I cleavage efficiency. Such a negatively acting sequence is most probably located on the *C. reinhardtii* cDNA, since the same plasmid vector was used for both the 31 bp and the 620 bp cDNAs. Consequently this putative negative element should not be present in the *Mycoplasma* clone, which was also a better substrate than the 620 bp *C. reinhardtii* cDNA, although the recognition sequences in both of these substrates are embedded in the context of an LSU ribosomal RNA sequence.

Extensive mutational analysis of the recognition sequences of intron-encoded DNA endonucleases has been reported for I-*Sce*I and I-*Sce*II. Sequence requirements for I-*Sce*I are quite strict since most single point mutations within the 18 bp recognition sequence strongly decrease or abolish cleavage efficiency (Colleaux et al. 1988). I-*Sce*II on the other hand tolerates many different single point mutations within its 18 bp recognition sequence (Sargueil et al. 1990; Wernette et al. 1992). The limited number of natural variants of the I-*Cre*I recognition sequence analysed in this study indicates that I-*Cre*I tolerates single and even multiple base-changes within its recognition sequence. This suggests that I-*Cre*I has a rather relaxed sequence specificity and that I-*Cre*I therefore is more similar to I-*Sce*II than to I-*Sce*I. However, precise determination of the size and positioning of the I-*Cre*I recognition sequence and the degree of sequence specificity of I-*Cre*I has to await extensive mutational analysis of the recognition sequence.

It has been proposed that the presence of highly homologous group I introns at the same position of the mitochondrial *coxI* genes (cytochrome *c* oxidase subunit 1) of the distantly related fungi *Schizosaccharomyces pombe* and *Aspergillus nidulans* is the result of a horizontal gene transfer event (Lang 1984). Homing endonucleases mediating such a horizontal gene transfer of group I introns would require a relaxed sequence specificity in order to cleave the heterologous homing sites. Here we show that I-*Cre*I cleaves ribosomal LSU DNAs from distantly related species *in vitro*, suggesting that the *C. reinhardtii* ribosomal intron has the potential to undergo analogous horizontal transfer.

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